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NON-MONOTONIC DOSE RESPONSES IN STUDIES OF ENDOCRINE DISRUPTING CHEMICALS: BISPHENOL A AS A CASE STUDY

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□ Non-monotonic dose response curves (NMDRCs) have been demonstrated for natural hormones and endocrine disrupting chemicals (EDCs) in a variety of biological systems including cultured cells, whole organ cultures, laboratory animals and human populations. The mechanisms responsible for these NMDRCs are well known, typically related to the interactions between the ligand (hormone or EDC) and a hormone receptor. Although there are hundreds of examples of NMDRCs in the EDC literature, there are claims that they are not 'common enough' to influence the use of high-to-low dose extrapolations in risk assessments. Here, we chose bisphenol A (BPA), a well-studied EDC, to assess the frequency of non-monotonic responses. Our results indicate that NMDRCs are common in the BPA literature, occurring in greater than 20% of all experiments and in at least one endpoint in more than 30% of all studies we examined. We also analyzed the types of endpoints that produce NMDRCs *in vitro* and factors related to study design that influence the ability to detect these kinds of responses. Taken together, these results provide strong evidence for NMDRCs in the EDC literature, specifically for BPA, and question the current risk assessment practice where 'safe' low doses are predicted from high dose exposures.

Keywords: biphasic, U-shaped, molecular mechanism, extrapolation, NOAEL, reference dose

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a class of chemicals that interfere in some way with the normal functioning of the endocrine system (Zoeller *et al.*, 2012). They include chemicals that can affect the synthesis, secretion, transport, binding or metabolism of hormones, as well as chemicals that can mimic or block the actions of hormones (Kavlock *et al.*, 1996). EDCs have recently received extensive attention from the scientific and regulatory communities. In 2013, the World Health Organization and United Nations Environment Programme updated their assessment of the science of EDCs, noting that there are a number of characteristics that should be expected from this class of chemicals (WHO, 2013). One of these characteristics was the ability of EDCs, like natural hormones, to produce non-monotonic dose response curves (NMDRCs). Similar conclusions about the ability of EDCs to produce NMDRCs have been made by the Endocrine Society, the director of the

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National Institutes of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP), and other scientists (Birnbaum, 2012; Myers *et al.*, 2009b; Vandenberg *et al.*, 2012; Vandenberg *et al.*, 2013a; Zoeller *et al.*, 2012).

NMDRCs are defined mathematically by a response where the slope of the curve changes sign from positive to negative, or vice versa, somewhere along the range of doses examined (Kohn and Melnick, 2002). The simplest way to define a NMDRC is by mathematically or statistically assessing changes to the sign (negative or positive) of the slope(s) of the dose response curve. Yet, most studies designed to assess dose responses do not identify the slope of the curve, nor do they calculate changes in the sign of the curve to verify non-monotonicity. Instead, there are numerous other ways that scientists determine whether non-monotonicity is manifested (reviewed in (Do *et al.*, 2012)). NMDRCs are distinct from another phenomenon that has received significant attention related to EDCs, i.e. the concept of "low dose effects." Low dose effects are defined as any biological changes occurring in the range of typical human exposures, or biological changes that occur at doses below those used in traditional toxicology studies (Melnick *et al.*, 2002). By definition, low dose effects make no assumption about the shape of the dose response curve. There is strong evidence that low dose effects exist and are reproducible for a number of EDCs (Birnbaum, 2012; Diamanti-Kandarakis *et al.*, 2009; Melnick *et al.*, 2002; Vandenberg *et al.*, 2012; Vandenberg *et al.*, 2013a; WHO, 2013; Zoeller *et al.*, 2012).

There are numerous mechanisms that have been described for the production of NMDRCs following exposure to or treatment with hormones. One well-studied mechanism is cytotoxicity, where increasing doses of a chemical induce an effect (i.e. cell proliferation) but also induce toxicity (Welshons *et al.*, 2003). Importantly, there are also examples where high doses of hormone inhibit cell proliferation – an effect that is distinct from cytotoxicity or toxicity in general (Sonnenschein *et al.*, 1989; Soto *et al.*, 1999). Thus, an inverted U-shaped curve can be observed when two competing monotonic curves overlap to produce an effect that manifests as a NMDRC – i.e. proliferation versus cell death, or proliferation versus inhibition of proliferation. Because many of the endpoints that are examined in biological studies are complex, it is not unexpected that NMDRCs can manifest due to complex interactions of many different responses at several levels of biological organization (see for example Angle *et al.*, 2013; Shioda *et al.*, 2006).

Another well-studied mechanism for non-monotonicity is the effect of hormone concentration on receptor number (Ismail and Nawaz, 2005; Nawaz *et al.*, 1999). When the number of receptors being produced does not equal the number of receptors being degraded, the response can decrease as the hormone concentration increases (von Zastrow and

Kobilka, 1994). For some receptors, the response at higher doses is also affected by desensitization, a process whereby receptors are biochemically inactivated when hormone concentrations increase (Freedman and Lefkowitz, 1996; Lohse, 1993). Other mechanisms that produce NMDRCs include cell and tissue specific receptors and co-factors (Jeyakumar *et al.*, 2008; Maffini *et al.*, 2008), receptor selectivity (McLachlan *et al.*, 2001; Sohoni and Sumpter, 1998; Tilghman *et al.*, 2010), receptor competition (Kohn and Melnick, 2002), and endocrine negative feedback loops (Bruchovsky *et al.*, 1975; Lesser and Bruchovsky, 1974; Stormshak *et al.*, 1976).

In addition to the acceptance of NMDRCs by endocrinologists, these responses are well understood by scientists in fields such as nutrition science where it is established that U- and inverted U-shaped curves exist for a number of nutrients. For example, too much or too little vitamin A is known to produce dermatitis, dementia and death, thus the optimal range of vitamin A consumption is in the mid-range. Similarly, NMDRCs are well understood by pharmacologists and are often referred to as "flare" (Harvey, 1997; McLeod, 2003). Yet traditional toxicology relies on the centuries-old dogma that "the dose makes the poison", and thus higher concentrations of a chemical are expected to have larger effects (Myers *et al.*, 2009b; vom Saal and Myers, 2010; Welshons *et al.*, 2006; Welshons *et al.*, 2003). In fact, although simplified, this is how regulation of chemicals is currently conducted: high doses of chemicals are tested to produce LD50s (the lethal dose at which 50% of the animals die), maximum tolerated doses (MTDs, or doses where no *unacceptable levels* of toxicity are observed), LOAELs (the lowest observed adverse effect level) and NOAELs (the no observed adverse effect level). For many chemicals, including many EDCs, these doses are in the milligram per kilogram body weight range (Vandenberg *et al.*, 2012). One or more safety factors are then applied to the NOAEL to calculate a reference dose, which is assumed to be safe for humans or wildlife. Yet this 'safe' dose is rarely tested – based solely on the assumption that there are monotonic relationships between dose and effect, and thus no adverse effects can occur below the NOAEL. If, however, there are non-monotonic relationships between dose and effect, this entire high dose testing paradigm is flawed. Furthermore, if NMDRCs are rare for EDCs and other toxicants then the premise of high dose testing to extrapolate to low 'safe' doses might be a satisfactory approach for the regulation of these chemicals. However, if NMDRCs are common, they challenge this *status quo*.

It has been asserted that NMDRCs are 'common' for EDCs – a determination that was made based on a substantial assessment of the EDC literature that uncovered NMDRCs for more than 70 EDCs, in cultured cells, laboratory animals and even human populations (Vandenberg *et al.*, 2012). Yet this statement was questioned because the frequency of

NMDRCs in relation to the total number of dose response curves that have been produced remains unknown (Rhomberg and Goodman, 2012). Studies of the general toxicology literature indicate that NMDRCs may occur in more than 10% of all dose responses (Davis and Svendsgaard, 1994); studies specifically focusing on hormetic curves, a type of NMDRC associated with low dose stimulation and high dose repression of effects, have reported similar frequencies (Calabrese *et al.*, 2007).

One EDC, bisphenol A (BPA), has been studied extensively in recent years, and its ability to produce NMDRCs has been debated. One reason BPA has received significant attention is its estrogenic activity *in vitro* and *in vivo* (Richter *et al.*, 2007; vom Saal *et al.*, 2007; Wetherill *et al.*, 2007); BPA was also identified as a high priority chemical in a toxicological screen performed by the NTP because of its ability to bind to a number of other receptors (Reif *et al.*, 2010). Human exposure to BPA is widespread (Geens *et al.*, 2012; Vandenberg *et al.*, 2010), and a relatively large number of epidemiology studies suggest relationships between BPA exposure levels and disease outcomes including cardiovascular disease, diabetes, obesity, and abnormal neurobehaviors (Braun and Hauser, 2011; Vandenberg *et al.*, 2013b). In 2007, an expert panel assembled by NIEHS concluded that there was strong evidence that BPA can produce NMDRCs (Wetherill *et al.*, 2007); however, that group did not attempt to quantify the frequency of NMDRCs in the BPA literature. To begin to address this issue, a pilot study was performed to determine the frequency of NMDRCs in a subset of the EDC literature, specifically *in vitro* studies of BPA. BPA was selected because of the large number of available studies; several hundred have been conducted in the past decade alone. Here, 109 *in vitro* studies were examined and the shapes of the dose response curves in the 388 experiments within these studies were characterized. The results of this pilot study indicate that NMDRCs are, in fact, common, and that factors associated with experimental design may influence the ability to observe these responses.

METHODS

Identification of *in vitro* BPA studies

A previous review of the BPA *in vitro* literature concluded that there was strong evidence for NMDRCs (Wetherill *et al.*, 2007). We identified all *in vitro* studies that had been conducted since that publication using the search engine PubMed and the search terms "bisphenol A" or "BPA". Each study was visually inspected to determine whether it included *in vitro* experiments. Only complete studies were included; several abstracts were removed from the database due to lack of experimental detail. Only studies published in English were included.

All studies were added to a single database, regardless of the number of doses examined or any other study characteristics. Information inputted from the published studies included: the cell line or cell type that was used, the origin (species) of those cells, the endpoint assessed, the lowest observed effective concentration (LOEC), all doses that were tested, and which doses were found to be significantly different from controls according to the study authors. Additional information was determined based on what was reported in the publication: the total number of doses tested, the number of log doses tested, the log span of the range of the doses tested, and the presence of NMDRCs.

Determination of non-monotonicity

Non-monotonicity is defined as a response with a change in the sign of the slope over the dose range tested. We did not identify a single study that calculated the slopes of dose response curves. Instead, we used the following criteria:

1. Visual inspection of the curve, especially when no statistics had been performed for specific doses.
2. Statistical analysis in the manuscript indicates that lower doses produce significant effects compared to untreated controls, but higher doses do not.
3. Statistical analysis indicates that lower doses produce an increase in response compared to untreated controls and high doses produce a decrease in response compared to untreated controls, or vice versa.
4. Visual inspection suggests a U- or inverted U-shaped curve is present and statistical analysis indicates that higher doses are significantly different from the response at lower doses, regardless of whether the response at the higher doses is significantly different from what occurs in untreated controls.

Analysis of monotonic and non-monotonic curves

109 *in vitro* BPA studies were identified as published between April/May 2007 (when the previous review of the *in vitro* literature was published) and April 2013. Studies were eliminated from further analysis if they examined only a single dose, or examined only those endpoints that were unaffected by BPA at any concentration. Of the remaining studies, the total number of doses were counted in each experiment and compared between endpoints reporting NMDRCs and those reporting monotonic response curves using a student's T-test. To account for the fact that some studies examine a large number of doses over a relatively small span of concentrations (1-2 log M), *the number of log doses* tested was determined for each experiment and compared between endpoints

reporting NMDRCs and those reporting monotonic response curves using a student's T-test. Finally, because some studies examine doses over a large span of concentrations (5 or more log M) but with gaps in the concentrations studied (i.e. testing 10^{-14} M and 10^{-12} M – two doses that span 3 log M), *the log range of doses* tested was determined for each experiment and compared between endpoints reporting NMDRCs and those reporting monotonic response curves using a student's T-test.

Each study was examined and if any endpoint displayed non-monotonicity according to the criteria listed above, the study was considered 'positive' for NMDRCs. The frequency of studies with NMDRCs was calculated by dividing the number of positive studies by the total number of studies.

Each experiment (examining a single endpoint) was also examined and if the experiment displayed non-monotonicity according to the criteria listed above, the experiment was considered 'positive' for NMDRCs. The frequency of experiments with NMDRCs was calculated by dividing the number of positive experiments by the total number of experiments.

Finally, to determine whether the number of doses, log doses or range of doses influenced the conclusions reached about non-monotonicity, Chi Square tests were compared between the entire dataset (250 experiments) and subsets of the dataset (i.e. studies only examining 'n' number of doses) where the frequency of non-monotonicity observed in the entire dataset was the 'expected' frequency and the frequency observed in the subset was the 'observed' frequency. For all comparisons, results were considered significant at $p<0.05$.

Analysis of Endpoints

For each study and each experiment, the endpoint that was examined was recorded. To determine whether certain endpoints were more likely to demonstrate NMDRCs, endpoints that were examined in multiple studies were identified and analyzed for the frequency of NMDRCs.

RESULTS

A database of *in vitro* studies of BPA: 2007-2013

We began by creating a database of all *in vitro* studies of BPA that had been published since the 2007 Chapel Hill expert panel review of the *in vitro* literature (Wetherill *et al.*, 2007). A total of 109 studies were identified with 388 experiments (Supplemental Table 1). These studies were examined individually, and any studies that only examined single doses in all experiments were removed, as single doses are incapable of providing information about the shape of the dose response curve. Also, studies that showed no effect of BPA on any endpoint examined were removed. Thus, a total of 93 studies with 250 experiments providing dose response

data were retained in the final dataset. Figure 1 shows the characteristics of these 250 experiments.

NMDRCs are common in the BPA *in vitro* literature

Once the 250 appropriate dose response experiments were identified, they were examined visually and the statistics performed by the original study authors were studied to determine whether each experiment met the criteria for non-monotonicity. Including those experiments that had not performed statistical analyses – and thus relying solely on the visual appearance of the dose response curve – a total of 59 experiments displayed NMDRCs, a total of 23.6%. Similarly, we examined the 93 studies that included at least one dose response experiment and asked whether a NMDRC was reported for any endpoint within that study. 32 studies (34.4%) included at least one experiment with a NMDRC.

To ensure that the visual inspection of studies did not influence the interpretation of non-monotonicity, we removed from the dataset any studies that did not include statistical analyses comparing the individual doses to the untreated control group. Following this exclusion, a total of 229 experiments were included, of which 53 fit the criteria for non-monotonicity (23.1%). From these analyses, it is clear that NMDRCs are widespread within this subset of the BPA *in vitro* literature.

Studies that report NMDRCs examine more doses, over wider ranges, than studies that do not

To determine whether factors associated with study design were associated with the detection of NMDRCs, we compared three measures in experiments with non-monotonic responses and experiments with monotonic responses. Experiments reporting NMDRCs examined on average 6.9 doses, whereas experiments that failed to detect NMDRCs examined only 4.6 doses (Figure 2A). Following a correction for studies that examined multiple doses within a small range of concentrations, we observed that experiments reporting NMDRCs examined an average of 5.6 log doses, whereas experiments that failed to detect NMDRCs examined on average only 3.9 log doses (Figure 2B). Finally, considering that many studies examined a small number of doses over a large range, we found that studies reporting NMDRCs included doses spanning 6.1 log M, whereas experiments that failed to detect NMDRCs included doses spanning only 4.5 log M (Figure 2C).

We also determined whether study size affected the probability of detecting a non-monotonic response. We examined the same three factors (number of doses, number of log doses, and span of log doses) and assessed the probability that a study with a particular design would display NMDRCs. In the total dataset, 23.6% of studies had NMDRCs. Smaller studies (those that examined only 2 doses, 2 log doses, or doses that only

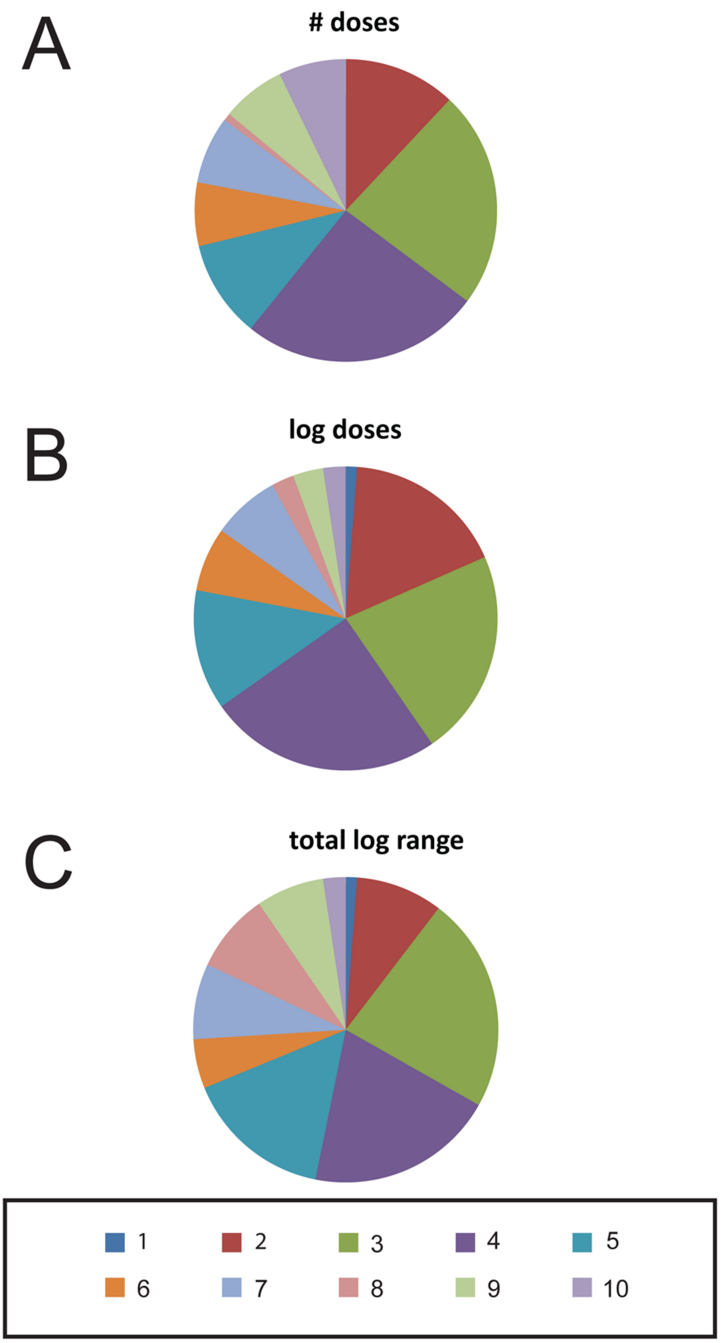

FIGURE 1. Characteristics of the 250 experiments identified for additional analyses. A) Graph illustrating the breakdown of the number of doses examined in these studies. The majority of studies examined only 2, 3 or 4 different concentrations of BPA. B) Graph illustrating the number of different log doses examined in these studies. This graph shows that the majority of studies examined doses from 2, 3 or 4 log M. C) Graph illustrating the span of log doses from the highest to lowest concentrations examined in these studies. Most studies examined doses that spanned 2-5 log M.

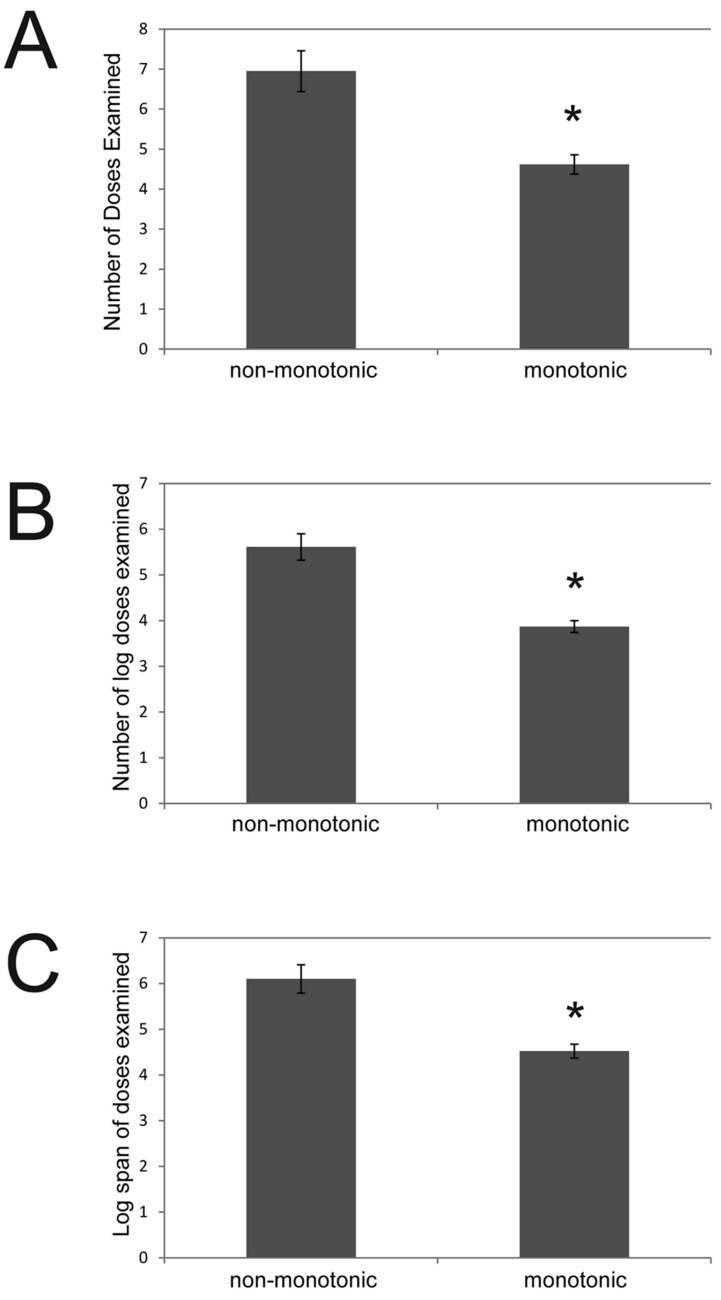

FIGURE 2. Comparisons of characteristics in experiments that report NMDRCs versus those that report monotonic responses. A) Comparisons of the number of doses examined. B) Comparisons of the number of different log doses examined. C) Comparisons of the span of log doses examined. All graphs represent means ± SEM, *p<0.05, T-test.

spanned 2 log M) were significantly less likely to report non-monotonic responses. Large studies (those that examined 7-10+ doses, or a wide log range) were significantly more likely to report non-monotonic responses (Figure 3). Collectively, these results are consistent with the conclusion that the inclusion of more doses in a study increases the ability to detect NMDRCs, and that studies that only examine a few doses over a small concentration range are more likely to report that an endpoint has a monotonic response.

NMDRCs occur for many different endpoints

A number of different endpoints manifesting at the subcellular and cellular levels of biological organization demonstrated NMDRCs. These responses were observed in the expression of certain genes and proteins, in the secretion of hormones and other substances, in chromosomal abnormalities, in cell proliferation and viability, in the phosphorylation of target proteins, in several types of mitochondrial responses, and in the accumulation of lipids (Supplemental Table 1).

Previous discussions of the non-monotonicity literature have suggested that NMDRCs are not reproducible, and therefore are not a concern (Rhomberg and Goodman, 2012). To assess this conclusion, we identified studies that examined the same or similar endpoints. In one analysis, we examined 28 experiments from 24 different studies that assessed the effects of BPA on cell proliferation (Supplemental Table 2). In some studies, cell proliferation was unaffected by any concentration of BPA that was tested (for example (Dominguez *et al.*, 2008)). In other studies, only monotonic responses were reported for cell proliferation (for example (Kim *et al.*, 2007; Kochukov *et al.*, 2009; Okada *et al.*, 2008; Park *et al.*, 2009)). Finally, still other studies reported non-monotonic responses for cell proliferation (for example (Bouskine *et al.*, 2009; Ricupito *et al.*, 2009; Sheng and Zhu, 2011; Wu *et al.*, 2012)). Yet, these studies should not be considered replicates of each other; they typically utilized different cell types, examined different doses, and utilized different assays to assess cell proliferation. Similar variability was observed for studies assessing cell viability, release/secretion of hormones, and gene expression, among others (Supplemental Table 1). Thus, assumptions that these NMDRCs are not reproducible is a conclusion that may reflect the diversity of cell lines and experimental conditions rather than a true assessment of whether a NMDRC observed in one cell type under specific conditions can be observed in multiple laboratories.

DISCUSSION

This pilot study examined a small portion of the EDC literature and concluded that NMDRCs were observed in more than 30% of all studies and for more than 20% of all experiments in the dataset examined.

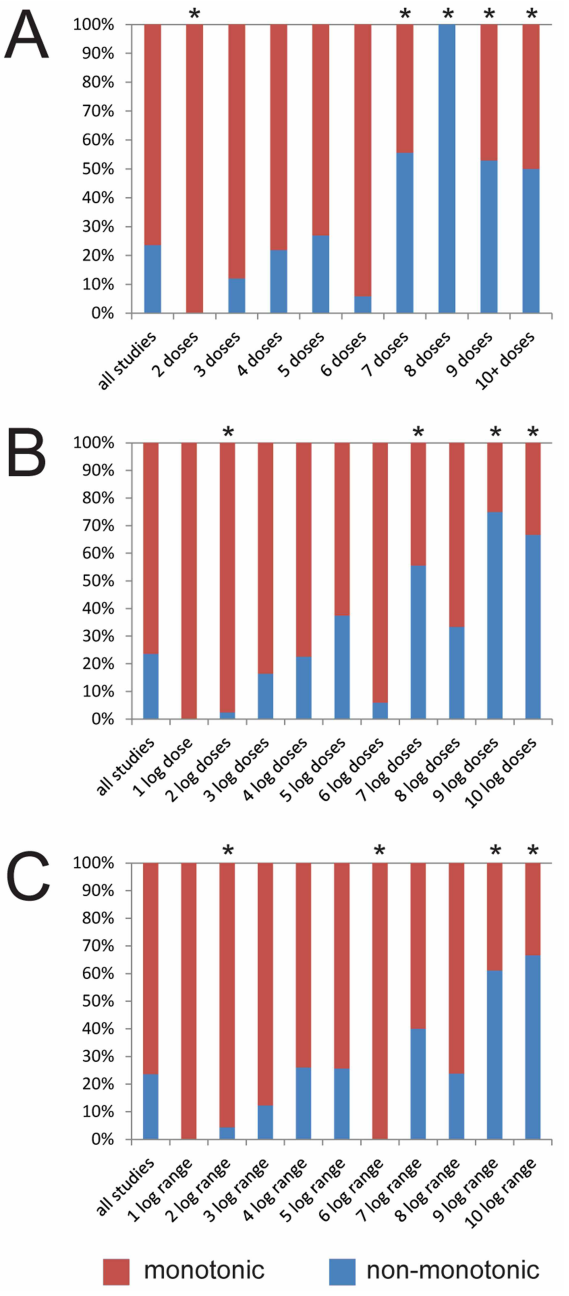

FIGURE 3. Percent of experiments displaying monotonic or non-monotonic responses depend on experimental design factors. A) Experiments were analyzed based on the number of doses examined and the percent displaying monotonic and non-monotonic responses is shown. B) Experiments were analyzed based on the number of different log doses tested and the percent displaying monotonic and non-monotonic responses is shown. C) Experiments were analyzed based on the range of log doses examined and the percent displaying monotonic and non-monotonic responses is shown. In all panels, each group was compared as an 'observed' value to the total number of experiments (59 non-monotonic, 191 monotonic; the 'expected' value) using a Chi Square test; *$p<0.05$.

Although these frequencies are likely to relate specifically to *in vitro* studies of BPA rather than to the wider EDC literature, this quantitative analysis provides support for statements that NMDRCs are 'common'. Furthermore, these frequencies are similar to what has been reported for NMDRCs and hormetic responses in the general toxicology literature. One study, examining a relatively small toxicology database, concluded that NMDRCs were present in 12-24% of all dose response studies, and further proposed that this could be an underestimate due to biases against studies that do not report monotonic responses (Davis and Svendsgaard, 1994). Other studies report non-monotonic hormetic responses in more than 20% of toxicology studies (Calabrese and Baldwin, 2001a; Calabrese and Baldwin, 2001b; Calabrese and Baldwin, 2001c). Thus, our results are consistent with other quantitative assessments of NMDRCs.

Our analysis also indicated that studies that identified NMDRCs examined more doses, over a wider range of concentrations, compared to studies that did not observe NMDRCs (Figure 2). This finding supports the use of a large number of doses, including doses that extend below the picomolar range, which have been successfully studied in numerous laboratories (see for example (Vinas and Watson, 2013)); experimenters should continue to investigate the effects of chemicals at these very low concentrations. There have been claims that reports of NMDRCs are flawed due to their use of too few doses to make appropriate conclusions about the shape of the dose response curve, i.e. suggestions that NMDRCs are simply 'statistical flukes' (Rhomberg and Goodman, 2012). This claim is not supported by data within this dataset; in fact, the data suggests that some claims of *monotonicity* may be flawed due to the selection of too few doses over a too small range of concentrations.

NMDRCs can occur across any point in the range of concentrations tested, from the true "low dose" range up to the high pharmacological range. For BPA, there is strong debate over what the "low dose" range might be, especially because the NTP's two definitions for low dose (typical human exposure levels or doses below the NOAEL) are quite divergent (~5μg/kg/day versus 50 mg/kg/day, respectively). For *in vitro* studies, the cut-off for a low dose is even more difficult to establish. Wetherill and colleagues (2007) set the cut-off at 1×10^{-7} M based on calculations of circulating BPA concentrations in animals administered 50 mg/kg/day (Vandenberg *et al.*, 2007; Welshons *et al.*, 2006). Others have suggested that the cut-off should be in the range of 1×10^{-9} M, based on the results of dozens of biomonitoring studies that measured BPA in human blood samples in the range of 0.1 – 2 ng/ml ($0.4 – 8.8 \times 10^{-9}$ M) (Vandenberg *et al.*, 2010). Still other scientists have disputed these biomonitoring studies and suggest that circulating levels of BPA in humans are in the range of 10^{-12} M (Dekant and Volkel, 2008; Teeguarden *et al.*, 2012; Ye *et al.*, 2013).

What is clear from our analysis of the BPA *in vitro* literature is that biological effects are observed at all of these low-dose cut-offs, and even more importantly, that NMDRCs are observed for some endpoints at doses that include or span these cut-off doses as well.

It has also been suggested that NMDRCs are only relevant if they are observed for 'adverse' endpoints. Although this is not directly relevant to the current study, as *in vitro* endpoints are rarely acknowledged to represent adverse endpoints, there is certainly evidence that effects observed in *in vitro* studies are relevant to *in vivo* endpoints (see for example (Wang *et al.*, 2012)), and there have been strong recommendations that results from *in vitro* studies be considered relevant to effects that are *likely to be observed* at other levels of biological organization (Schug *et al.*, 2013). Furthermore, the development of Tox21, ToxCast, and other high throughput *in vitro* assays acknowledges that *in vitro* cell based assays and cell-free assays provide important tools that can and should inform chemical risk assessments (Knudsen *et al.*, 2013; Mahadevan *et al.*, 2011). These assays have many benefits over *in vivo* tests, including the ability to test large numbers of doses over a large span of log concentrations, their high sensitivity and specificity, and their use of state-of-the-art techniques (Shukla *et al.*, 2010; Sun *et al.*, 2012). In contrast, there is an extensive ongoing discussion about whether the endpoints examined in traditional (*in vivo*) guideline studies are sufficient to capture the effects of EDCs on reproduction and development, and whether the endpoints in these assays reflect current knowledge of endocrine toxicity (Zoeller *et al.*, 2012). There have been calls to expand the endpoints involved in the assessment of EDCs to include more sensitive endpoints than the ones currently used (Myers *et al.*, 2009a; Myers *et al.*, 2009b; Vandenberg *et al.*, 2013a; vom Saal *et al.*, 2007; vom Saal *et al.*, 2010; vom Saal and Myers, 2010).

Importantly, guideline studies – studies that follow internationally agreed-upon methods to assess toxicity and endocrine disrupting properties of test chemicals – typically examine only three doses. In some historical assays (i.e. those found in the NTP's carcinogenesis database), only two doses of the test chemical were examined. This may be one reason why guideline studies overwhelmingly fail to detect NMDRCs, which some scientists have interpreted to mean that NMDRCs do not exist in these studies, or do not exist for the kinds of endpoints assessed in these studies, which are widely acknowledged to be adverse (Rhomberg and Goodman, 2012). The presence (or absence) of NMDRCs from guideline studies is an important issue, as these are the studies that regulators rely on heavily when making decisions about chemical safety and in setting "safe" reference doses (Tyl, 2009). Yet analyses of guideline studies indicate that NMDRCs are present, but are often ignored or dismissed as paradoxical or irrelevant (Patisaul *et al.*, 2012; Vandenberg *et al.*, 2013a).

This study was designed to determine the frequency of NMDRCs in a small portion of the EDC literature, specifically focusing on a single well-studied EDC. Additional studies are needed to assess the occurrence, frequency, and implicated endpoints for NMDRCs in *in vivo* studies of BPA and for other EDCs. NMDRCs have been reported for BPA in a number of studies of laboratory animals (see for example (Angle *et al.*, 2013; Ayyanan *et al.*, 2011; Cabaton *et al.*, 2011; Jenkins *et al.*, 2011; Marmugi *et al.*, 2012; Xu *et al.*, 2010)), but the majority of studies examining rodents or aquatic animals have used fewer than six doses (Richter *et al.*, 2007; vom Saal and Hughes, 2005; Welshons *et al.*, 2006), and thus NMDRCs are likely to be more difficult to observe or detect.

In conclusion, this pilot study of the BPA *in vitro* literature illustrated that NMDRCs occur in more than 1/5 of experiments and in more than 1/3 of studies that were appropriately designed to assess dose responses (i.e. studies that examined more than one dose and identified at least one endpoint affected by BPA.) This study is a first step toward quantifying the frequency of NMDRCs in the EDC literature. Global chemical regulation practices rely on an assumption of monotonicity, where it is expected that high dose testing, coupled with the use of safety factors, can identify safe dose ranges for humans and wildlife (Fenner-Crisp, 2000; Lucier, 1997; Sheehan and vom Saal, 1997). The presence of NMDRCs challenges the use of high-to-low dose extrapolations as well as the use of a threshold model, which proposes that there is a dose below which no effects of a chemical are observed (Calabrese and Baldwin, 2003; Cook and Calabrese, 2006; Sheehan, 2006; vom Saal and Sheehan, 1998). This study provides another step toward challenging the *status quo* in risk assessment, indicating that NMDRCs occur frequently enough that they should not be ignored.

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